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Sampling considerations pertinent to the detection of analytes in swine oral fluids

by

Christopher Wayne Olsen

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Veterinary Preventative Medicine

Program of Study Committee:
Jeffrey J. Zimmerman, Co-Major Professor
Derald J. Holtkamp, Co-Major Professor
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Iowa State University
Ames, Iowa
2012

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ABSTRACT

Surveillance for swine pathogens and health related biomarkers is of great importance to the financial success of the swine industry and the health of animal and human populations worldwide. Research has shown that the use of oral fluid specimens in diagnostics and surveillance provides many advantages over serum. To discover the full potential of this technology many aspects of its use must be evaluated e.g. sample collection and handling techniques, assay performance parameters and detection limits of pen-based samples.

As reviewed in Chapter 1, the detection of many analytes in human and animal oral fluid samples has been reported. In the process of these discoveries, it was been identified that oral fluid sample collection material, post-collection processing and sample storage can affect testing results of human and animal oral fluid samples. Though little data was found in the literature regarding these effects on animal samples, limited reports suggest they may similarly affect veterinary testing of oral fluid specimens. As more diagnostic tests for human and animal oral fluid samples become available, it will be important to consider if and how these external factors affect testing results.

Pursuant to the concerns identified in Chapter 1, the objective of the first research project (Chapter 2) was to evaluate the effect of oral fluid sampling material and post-collection processing on the results produced by antibody- and polymerase chain reaction-based assays. Oral fluid samples were collected from 104 pens of commercial wean-to-finish pigs using 3 types of rope. Processed (centrifuged and filtered) and unprocessed oral fluid samples were tested using commercial ELISAs for porcine reproductive and respiratory syndrome virus (PRRSV) antibodies and total IgM, IgA, and IgG. Only unprocessed samples were tested for PRRSV nucleic acid and processed samples for PRRSV neutralizing antibodies. It was found that all three factors could affect results, but statistically significant effects were non-uniform and assay-dependent.

The objective of the second study (Chapter 3) was to evaluate the detection limits of assays detecting PRRSV antibodies and PRRS virus in pen-based oral fluid samples from commercial swine. Five successive oral fluid samples were collected from 25 pens of commercial swine of known PRRSV prevalence (0%, 4%, 12%, 20% and 36%) with five

pens per prevalence level. PRRSV prevalence was established by vaccinating commercial swine with an intramuscular modified live PRRSV vaccine 14 days prior to the trial. The vaccinated pigs were then introduced at the designated prevalence levels into pens of otherwise PRRSV negative pigs one day prior to sample collection. Serum was collected from each pig in each pen to confirm expected PRRSV prevalence. Oral fluid and vaccinated pig serum was tested using RT-PCR and ELISA by six laboratories. Intra-laboratory agreement was measured and predicted probability of PRRSV detection in pen-based oral fluid samples was estimated for each assay.

INTRODUCTION: THESIS ORGANIZATION

This thesis is organized in four chapters. Chapter 1 contains a general introduction to the thesis organization and a literature review. The literature review is titled "The influence of external factors on the results of oral fluid testing" and will be submitted to the Journal of Animal Health Research Reviews for publication. Chapter 2 is a scientific research paper titled "Effect of collection material on pig oral fluid testing results" and has been submitted Research in Veterinary Science for publication. Chapter 3 is the final scientific research project titled "Probability of detecting PRRSV infection in pen-based swine oral fluid samples". This chapter will be submitted for publication in the Journal of Veterinary Diagnostic Investigation. The final chapter contains general conclusions for the entire thesis.

CHAPTER 1. THE INFLUENCE OF EXTERNAL FACTORS ON THE RESULTS OF ORAL FLUID TESTING

For submission to Animal Health Research Reviews

Chris Olsen, Jeffrey J. Zimmerman

ABSTRACT

Oral fluid is widely used in human and veterinary diagnostics. Collection of oral fluid is convenient, economical, does not require special training and can be done on-site in both humans and animals. Because oral fluids reflect many of the bioanalytical substances found in serum and tissues and is a convenient sample it and has been the focus of significant investigation in humans and animals. Researchers have reported that external factors i.e. sample collection material, post-collection sample processing and sample storage can affect oral fluid testing results. Cotton collection materials have reportedly reduced the level of antibody, hormones, and total protein in oral fluid. Sample processing techniques such as centrifugation and/or filtration also reduced antibody, hormone and protein measurements. Analyte stability is important in all biological samples. Since oral fluid can be collected in the home or on the farm by untrained individuals, shipping samples is common. Many inconsistencies were identified for a variety of targets in oral fluid. In general, targets are stable at freezing temperatures and for short periods of time at refrigeration temperature. This review will outline the literature surrounding the effect of external factors on oral fluid testing and apply the data to current oral fluid usage in diagnostic medicine.

1. INTRODUCTION

The use of oral fluid specimens in disease diagnosis and health monitoring provides many advantages over serum and other sample types. As outlined in recent research reports and

reviews, collection of oral fluid is convenient, economical, does not require special training and can be done on-site in both humans and animals (Scully, 1997; Hofman, 2001; Gomes-Keller et al, 2006; Tabak, 2007; Prickett et al., 2008a, 2008b; Kittawornrat et al., 2010; Prickett and Zimmerman, 2010). More importantly, the detection of numerous bioanalytical targets in oral fluid has been reported in humans and animals (Table 1).

Oral fluid is comprised mainly of secretions from three pairs of major salivary glands (parotid, sublingual, and submandibular) but also contains components from other sources such as serum transudate, oro-naso-pharyngeal secretions, and gingival cervicular fluid (Delima and van Dyke, 2000; Aps and Martens, 2005; Cameron and Carman, 2005; Prickett and Zimmerman, 2010). Studies have shown that when fluorescein dye is injected into the hind leg of dogs, or ingested by humans it can be detected in the gingival cervicular fluid in as little as 30 seconds (Brill and Krasse, 1958; Brill and Björn, 1959). Because of the passage of transudates from serum and tissues into the oral cavity, it has been said that oral fluid is a "mirror of the body" (Mandel, 1993) and reflects many of the bioanalytical substances found throughout the body. Therefore it is no surprise that immunoglobulins (Brandtzaeg 2007), hormones (Vining et al, 1983), and drugs (Aps and Martens, 2005) have been reported to be present in oral fluid as a result of passive diffusion.

After many years of research, tests are now being utilized in diagnostic laboratories to test for targets in human and animal oral fluid. The first kit for collecting and diagnosing human immunodeficiency virus type 1 (HIV-1) antibody in human oral fluid was approved by the United States Food and Drug Administration (FDA) in 1994 (Nightingale, 1995). Since this time, several other oral fluid tests for HIV type 1 and 2 have been approved for human use (FDA, 2011). In addition to HIV, the detection of hormones in human saliva is routinely practiced and has been reported to be the second most researched topic in human oral fluid research (Malamud and Rodrigues-Chavez, 2011). In pigs, oral fluid is routinely used for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) and antibody (Kittawornrat et al., 2012; Ramirez et al., 2012), influenza virus (Detmer et al., 2011) and porcine circo virus type 2 (PCV2) (Ramirez et al., 2012). Oral fluid assays for the detection of feline leukemia virus in cats (Lewis et al., 1987; Gomes-Keller et al., 2006) and rabies virus in dogs (Kasempimolporn et al., 2011) have also been reported. As shown in Table 1,

many other pathogens, hormones, and other biomarkers in humans and animals can be detected in oral fluid samples.

As research surrounding the use of oral fluid developed, studies identified that testing results may be influenced by external factors. Collection material (Table 3), post-collection processing (Table 4) and sample storage conditions (Table 5) have been reported to affect testing results for antibodies, hormones or drugs. Little data regarding these topics in veterinary applications was found in the literature, but may similarly affect veterinary oral fluid testing. The purpose of this review was to examine and summarize reports that indicate collection material, sample processing and sample storage conditions affect oral fluid testing results.

2. TECHNIQUES FOR ORAL FLUID COLLECTION HANDLING AND STORAGE

Oral fluid can be collected via a variety of methods (Table 2). Four general techniques have been outlined for the collection of oral fluid samples from humans; draining, expectoration, suction, or absorption (Navazesh, 1993). Though expectoration is frequently used in human subjects (Kim et al., 2010, Williamson et al., 2012) in other circumstances, it is not desirable or is not possible e.g. with animal subjects. Consequently, absorptive materials are used to soak up fluids from the oral cavity (Prickett et al., 2008a, 2008b; Chang et al., 2009; Kittawornrat et al., 2010), and thereafter, extracted from the material and used for testing. Because of the variety in collection methods in animals and humans, it is important to accurately describe the resulting samples using standardized terminology. Following the guidelines outlined by Atkinson et al. (1993), the definitions for "whole saliva", "the fluid obtained…by expectoration" and "oral fluid", "the fluid obtained by insertion of absorptive collectors into the mouth" will be used throughout this review.

Samples can also be described as stimulated or unstimulated depending on the method of collection, or use of chemical stimulants to induce salivary flow (Navazesh, 1993; Schwartz et al., 1998; Bergeron et al., 2002). Samples collected with absorptive materials are often considered "stimulated", whereas samples obtained via expectoration or drooling are called "unstimulated" (Atkinson et al., 1993; Navazesh, 1993). For the purposes of the present

work, the main interest lies in the time in which the sample is in the collection material and forward. Therefore, stimulation of salivary flow is of less interest and will not be considered in this review.

Oral fluid is collected from animals using absorptive materials, i.e. a swab or by allowing the animals to chew on the collection material (Harley et al., 1998; Prickett et al., 2008a, 2008b; Dreschel and Granger, 2009; Kittawornrat et al., 2010). To collect oral fluid from cattle or pigs, collection material e.g. rope, is suspended in the pen, allowing animals to interact with the rope and deposit oral fluids (Smith et al., 2004, 2005a, 2005b; Prickett et al., 2008a, 2008b; Kittawornrat et al., 2010). In cats and dogs, swabs (Harley et al., 1998; German et al., 1998; Kasempimolporn et al., 2000) or dental rope (Dreschel and Granger, 2009) have been used to absorb oral fluid from the surfaces on the inside of the mouth. Based on the terminology used in this report, samples collected by these methods from animals will be considered oral fluid.

3. EFFECT OF COLLECTION MATERIAL ON TESTING RESULTS

The detection of pathogens and pathogen-specific antibodies has been widely used in human and veterinary diagnostics (Prickett et al., 2008a, 2008b; Pink et al., 2009; Kittawornrat et al., 2010; Delaney et al., 2011; Detmer et al., 2011; Malamud et al., 2011; Ramirez et al., 2011; Kittawornrat et al., 2012). Over the past few decades it has become evident that measurements of antibody in oral fluid samples can be influenced by the material used to collect the samples. Total immunoglobulin M (IgM), A (IgA), or G (IgG) measurements were reportedly reduced in association with cotton collection materials in human oral fluid specimens (Aufricht et al., 1992; Shirtcliff et al., 2001; Strazdins et al., 2005; Michishige et al., 2006; Chang et al., 2009). Chang et al., (2009) also reported a reduction of IgM, IgA and IgG in samples collected with polystyrene materials. In pigs, samples collected with cotton had lower concentrations of total IgM, IgA and PRRSV neutralizing antibody titers in comparison to samples collected with hemp or nylon (Olsen et al., 2012). IgG and PRRSV-specific IgG antibodies were not affected by any of the materials used in this study but detection of PRRS virus by RT-PCR was significantly reduced in samples collected with

hemp or nylon.

Measurement of hormones e.g. 17- hydroxyprogesterone (17-OHP) (Kruger et al., 1996), cortisol (Levine et al., 2007), DHEA (Johnson et al., 2002), testosterone (Dabbs et al., 1993), and progesterone (Gombe, 1977) in biological samples is a useful tool to monitor various psychological, developmental, physiological or health-related paradigms (Granger et al., 1999). Considerable evidence has suggested that assay results for the detection of hormones in human oral fluid are affected by the material used to collect the sample (Table 3). The reported effects of sampling material on assay results however, were not consistent. Measurements of cortisol were reportedly increased (Strazdins et al., 2005; Atkinson et al., 2008) or decreased (Shirtcliff et al., 2001; Gröschl et al., 2008; Hansen et al., 2008) in samples collected with cotton. Similarly, testosterone levels in oral fluid collected with cotton were increased (Shirtcliff et al., 2001; Granger et al., 2004; Atkinson et al., 2008) or decreased (Gröschl et al., 2008). Samples collected with polyester materials resulted in increased DHEA (Granger et al., 1999) and testosterone (Granger et al., 2004).

It is evident that there is a need to identify how the reported effects are occurring. Despite significant data identifying the influence material may have on assay results, little data could be found supporting a mechanism for the reported effects. Shirtcliff et al. (2001) hypothesized that analytes in the oral fluid sample may bind to fibers in cotton sampling materials. This however does not explain the increased levels of some hormones in samples collected with cotton. It was reported by another author that water absorbed onto four types of cotton, extracted, then assayed for testosterone resulted in significantly higher testosterone concentrations that water alone (Dabbs, 1991). In this report the author suggests that molecules exist within the cotton, either natively or through manufacturing processes, "that mimics the effect of testosterone when assayed" (Dabbs, 1991). Similar to hormones, it is largely unknown how sampling material affects the detection of antibodies, pathogens or other analytes in oral fluid samples.

It is important to consider the differences in experimental design when evaluating the reported effects of oral fluid collection material on assay results reported in Table 3. For example, some studies collected saliva from study subjects (pooled or individual), and

subjected aliquots to different treatments e.g. in vitro absorption via collection materials or collected multiple samples from each subject using different materials. Additionally some studies measured inherent concentrations of analytes in samples whereas others measured spiked concentrations of assay targets. One study (Chang et al., 2009) applied known concentrations of total human immunoglobulin diluted in phosphate buffered saline to oral fluid sampling devices to test recovery. The sample size for most human studies was relatively small (< 30 subjects), however, multiple studies have reported similar effects which support the validity of the reported data.

4. SAMPLE PROCESSING

Post-collection sample centrifugation is frequently practiced with animal and human oral fluid samples (Harmon et al., 2007; Atkinson et al., 2008; Prickett et al., 2008b; Kittawornrat et al., 2010) either for the purpose of harvesting the sample from a collection device or to clarify the sample. In veterinary medicine, centrifugation is often performed because many field collected oral fluid samples contain significant amounts of manure, feed and other environmental particles. Sample filtration has also been used to further remove particulate matter from oral fluid samples. Consequently, it is important to consider the impact such procedures may have on the detection of assay targets.

Little data was found regarding the effect of oral fluid processing on target detection. In the studies found, the general trend was that processing reduced the results of the respective assays (Table 5). In contrast, cortisol levels were reportedly increased in samples filtered through a 0.2 µm filter before testing in comparison to samples which were frozen, thawed and centrifuged at 10,000 x *g* for 10 minutes (Atkinson et al., 2008). In pig oral fluid samples, centrifugation (12,000 x *g* for 8 hours) significantly reduced the amount of PRRSV virus which had been spiked in the sample prior to centrifugation (Rotolo et al., 2012). Total IgM , IgA, and IgG concentrations were also reduced by processing (centrifugation at 10,000 x *g* for 2 hours followed by filtration through a 0.22 µm filter) pig oral fluid samples in comparison to unprocessed specimens (Olsen et al., 2012).

5. SAMPLE STORAGE

Sample storage is a critical factor for any biological material collected for diagnostic purposes. Oral fluid is increasingly used to gather large-scale epidemiological data (Frerichs et al., 1994; de Azevedo et al., 1995; Connolly et al., 2004; Nigatu et al., 2008) from humans, and for disease surveillance in pigs (Prickett et al., 2008a; Kittawornrat et al., 2012; Ramirez et al., 2012). Because such samples are commonly collected in the home or on the farm, samples often require shipment of samples to a laboratory for analysis. Short-term stability is especially critical in these situations to ensure samples arrive in optimal condition. Because oral fluid use is relatively new in diagnostics, the stability of many assay targets is unknown. Several reports were found regarding short-term storage of human oral fluid specimens, but little data was found for the stability of targets in animal samples (Table 4).

In regards to short-term storage, general trends were identified based on the data in Table 4. Measurements of total immunoglobulin in pig oral fluids appear to be stable for up to 12 days at temperatures ≤ 20 °C. At 30 °C, IgA and IgG isotypes were reduced after 2 days (Prickett et al., 2010). Cortisol measurements in human oral fluid samples were reportedly stable at room temperature or 4 °C for 5-7 days (Aardal and Holm, 1995; Clements and Parker, 2005; Garde et al., 2005). The reported effect of other storage conditions and assay targets was highly variable with no obvious trends.

As with studies comparing sampling materials, research methods in storage condition studies were highly variable and could not be reflected entirely in Table 4. Sample handling (centrifugation vs. no centrifugation), inconsistent collection methods, and variable freeze-thaw cycles and testing conditions are all potential sources of inconsistency among the data reported. Nevertheless, it is important to consider the reported effects in future studies of oral fluid use.

6. CONCLUSIONS

Interpretation of the reported data must be done cautiously because of the potential sources of variation identified. Despite inconsistencies, these data can be used as a guideline for future

research. Oral fluid continues to play an increasingly significant role in human (Hart et al., 2011; Malamud 2011) and veterinary diagnostics (Detmer et al., 2011; Kittawornrat et al., 2012; Ramirez et al., 2012). Many factors support the continuation of its use e.g. ease of collection, minimal stress on subject of collection, ability to obtain large sample numbers in addition to the performance of some diagnostic assays (Delaney et al., 2011; Kittawornrat et al., 2012). The data presented in this review identify areas of this science that may benefit from further research. Understanding the mechanisms by which sample collection materials influence assay results will help standardize methods and thereby aid in improved accuracy of assay results. Furthermore, additional data on how storage conditions and post-collection processing techniques impact target detection will ensure the integrity of the sample through all phases of its collection and use.

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Table 1. Select analytes detected in oral fluid specimens

Target	Analyte	Species	Citation
Pathogens			
Hepatitis A and B	IgG	Humans	Parry, 1987
HIV	IgA	Humans	Archibald et al., 1987
Influenza	IgA	Humans	Waldman, 1968
	Virus	Pigs	Detmer et al., 2011
CSFV	Antibody	Pigs	Corthier & Aynaud, 1977
PCV2	Virus	Pigs	Prickett et al., 2008b
PRRSV	Virus, IgM, IgA, IgG	Pigs	Prickett et al., 2008a, 2008b; Kittawornrat et al., 2010; Kittawornrat et al., 2012
FMDV	Virus, Neutralizing Antibody	Pigs	Eblé et el., 2004
	Neutralizing Antibody	Cattle	Figueroa et al., 1973
Escherichia coli	Bacteria	Cattle	Smith et al., 2004
Salmonella	Bacteria	Cattle	Smith et al., 2004b
FeLV	Virus	Feline	Lewis et al., 1987; Gomes-Keller et al., 2006
Rabies	Virus	Canine	Kasempimolporn et al., 2011
Hormones			
17-OHCS		Humans	Hoepffner and Hubl, 1986
Cortisol		Humans	Shannon et al., 1959
		Pigs	Ruis et al., 1987
DHEA		Humans	Finlay, 1982
Progesterone		Humans	Gombe, 1977
Testosterone		Humans	Gaskell et al., 1980

Table 1. (continued) Select analytes detected in oral fluid specimens

Target	Analyte	Species	Citation
Drugs			
Amphetamines		Humans	Wan et al., 1978
Barbiturates		Humans	Cook et al., 1975
Cocaine		Humans	Inaba et al, 1978
Marijuana		Humans	Just et al., 1974
Opiates		Humans	Leute et al., 1972
Other			
Antimicrobials		Pigs	Meiszberg et al., 2011
C-reactive protein		Humans	Christodoulides et al., 2005
		Pigs	Gutierrez et al., 2009; Gomez-Laguna et al., 2010
Haptoglobin		Pigs	Gomez-Laguna et al., 2010

* Abbreviations: HIV, human immunodeficiency virus; CSF, classical swine fever virus; PCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus; FMDV, foot-and-mouth disease virus; FeLV, feline leukemia virus; 17- OHCS, 17-hydroxycorticosteroid; DHEA, dehydroepiandrosterone

Table 2. Collection devices for collecting human oral fluid samples

Device	Format and Material	Manufacturer	Citation
Dental Rope	Cotton rope	Not reported	Dreschel et al., 2009
DentaSwabs®	Polystyrene sponge on plastic stem	Kimberly-Clark Corporation, Draper, UT USA	Chang et al, 2009
Dri-Angle®	Cellulose pad	Dental Health Products, Inc., Niagra Falls, NY USA	Chang et al, 2009
Gauze sponge	Layered cotton sponges	Henry Schein Inc., Melville, NY USA	Chang et al, 2009
No. 2 cotton roll	Cotton roll	Sullivan-Shein, Melville, NY USA	Chang et al, 2009
Omni-SAL	Absorptive pad on plastic stem	Saliva Diagnostic Systems, Vancouver, WA USA	Vyse et al., 2001
Oracol	Foam swab on plastic stem	Malvern Medical Developments, Worcester, UK	Vyse et al., 2001
Oragene™	Sponge swab	DNA Genotek Inc., Kanata, Ontario, Canada	Rogers et al., 2007
ORALscreen™	Foam rectangle	Avitar, Inc., Canton, MA USA	Barrett et al., 2001; Chang et al, 2009
OraSure®	Cotton pad on plastic stem	OraSure Technologies Inc., Bethlehem, PA USA	Chang et al., 2009
Saliva•sampler®	Cotton pad on plastic stem	Saliva Diagnostic Systems, Vancouver, WA USA	Langel et al., 2008; Chang et al, 2009
Salivette®	Untreated cotton roll	Sarstedt, Newton, NC USA	Granger et al., 1999
" "	Citric acid treated cotton roll	" "	Granger et al., 1999
" "	Polyester swab	" "	Granger et al., 1999
Toothette-Plus	Sponge on plastic stem	Sage Products Inc., Crystal Lake, IL USA	Holm-Hansen et al, 2004
UpLink®	Sponge on plastic stem	OraSure Technologies Inc., Bethlehem, PA USA	Holm-Hansen et al, 2004

Table 3. Effect of oral fluid sampling material on assay results in relation to a sample of reference from the same source

Target	Analyte	Species	Material	Reference Sample	Effect	Assay	Citation
Total_Antibody							
	IgM	Humans	Cotton	Spiked Amount	Decreased	ELISA	Chang et al., 2009
	" "	Humans	Polystyrene	Spiked Amount	Decreased	ELISA	Chang et al., 2009
	" "	Pigs	Cotton	Nylon	Decreased	ELISA	Olsen et al., 2012
	IgA	Humans	Cotton	Whole saliva	Decreased	EIA, ELISA	Aufricht et al., 1992; Shirtcliff et al., 2001; Strazdins et al., 2005; Michishige et al., 2006
	" "	Humans	Cotton	Spiked Amount	Decreased	ELISA	Chang et al., 2009
	" "	Humans	Polystyrene	Spiked Amount	Decreased	ELISA	Chang et al., 2009
	" "	Pigs	Cotton or hemp	Nylon	Decreased	ELISA	Olsen et al., 2012
	IgG	Humans	Cotton	Spiked Amount	Decreased	ELISA	Chang et al., 2009
	" "	Humans	Polystyrene	Spiked Amount	Decreased	ELISA	Chang et al., 2009
	" "	Pigs	Cotton	Hemp or nylon	No effect	ELISA	Olsen et al., 2012
Pathogens							
PRRSV	Neutralizing antibody	Pigs	Cotton	Hemp or nylon	Reduced	IFA	Olsen et al., 2012
	IgG	Pigs	Cotton or hemp	Nylon	No effect	ELISA	Olsen et al., 2012
	Virus	Pigs	Hemp or nylon	Cotton	Reduced	RT-PCR	Olsen et al., 2012
Other							
	Total protein	Human	Cotton	FTC	Decreased	CBQCA[a]	Atkinson et al., 2008
	" "	Human	Cotton	Whole saliva	Decreased	Lowry[b]	Michishige et al., 2006

Table 3. (continued) Effect of oral fluid sampling material on detection of analytes in relation to a sample of reference from the same source

Target	Analyte	Species	Material	Reference Sample	Effect	Assay	Citation(s)
Hormones							
	17-OHP	Human	Cotton	Whole saliva	Increased	RIA	Kruger et al., 1996
	Cortisol	Human	Cotton	Whole saliva	Increased	EIA, RIA	Strazdins et al., 2005 ; Atkinson et al., 2008
	" "	Human	Cotton	Spiked Amount	Decreased	EIA, LC-MS/MS	Shirtcliff et al., 2001; Gröschl et al., 2008
	" "	Human	Cotton	Polyester	Decreased	RIA	Hansen et al., 2008
	DHEA	Human	Cotton	Whole saliva	Increased	EIA , RIA	Granger et al., 1999; Shirtcliff et al., 2001; Atkinson et al., 2008
	" "	Human	Foam	Whole saliva	Decreased	EIA	Atkinson et al., 2008
	" "	Human	Polyester	Whole saliva	Increased	RIA	Granger et al., 1999
	Progesterone	Human	Cotton	Whole saliva	Increased	EIA	Shirtcliff et al., 2001
	Testosterone	Human	Cotton	Whole saliva	Increased	EIA, RIA	Shirtcliff et al., 2001; Granger et al., 2004; Atkinson et al., 2008
	" "	Human	Polyester	Whole saliva	Increased	RIA	Granger et al., 2004
	" "	Human	Cotton	Spiked Amount	Decreased	LC-MS/MS	Gröschl et al., 2008

* Abbreviations: ELISA, enzyme-linked immunosorbent immunoassay; EIA, enzyme immunoassay; PRRSV, porcine reproductive and respiratory syndrome virus; IFA, immunofluorescence assay; RT-PCR, reverse transcription polymerase chain reaction; 17-OHP, 17-hydroxyprogesterone; RIA, radioimmunoassay; LC-MS/MS, liquid chromatography with tandem mass spectrometry; DHEA, dehydroepiandrosterone; FTC, Freeze-thaw-centrifuge (4,000 x *g* for 10 min)

[a] 3-(4-carboxybenzoyl)quinoline-2-carboxalde-hyde (CBQCA) protein quantitation kit (Life Technoloties™, Foster City, CA USA)

[b] Protein quantitation via the methods described by Lowry et al., 1951

Table 4. Effect of post-collection sample processing on assay results

Target	Analyte	Species	Processing/Treatment	Reference Sample	Effect	Assay	Citation
Total Antibody	IgM	Pigs	Centrifuge - 10,000 x *g* 2 hrs and filter (0.22µm)	Unprocessed sample	Reduced	ELISA	Olsen et al., 2012
	IgA	Pigs	Centrifuge - 10,000 x *g* 2 hrs and filter (0.22µm)	Unprocessed sample	Reduced	ELISA	Olsen et al., 2012
	IgG	Pigs	Centrifuge - 10,000 x *g* 2 hrs and filter (0.22µm)	Unprocessed sample	No effect	ELISA	Olsen et al., 2012
Pathogens							
PRRSV	Virus	Pigs	Centrifuge - 12,000 x *g* 12 hrs	Unprocessed sample	Decreased	RT-PCR	Rotolo et al., 2012
	IgG	Pigs	Centrifuge - 10,000 x *g* 2 hrs and filter (0.22µm)	Unprocessed sample	No effect	ELISA	Olsen et al., 2012
Hormones							
Cortisol		Humans	0.2 µm filter	FTC sample	Increased	RIA	Atkinson et al., 2008
		Humans	Centrifuge - 10,000 x *g* 10 min	FTC sample	No effect	RIA	Atkinson et al., 2008
		Humans	0.22 µm filter	Unfiltered sample	Decreased	EIA	Whembolua et al., 2006
Testosterone		Humans	0.2 µm filter	FTC sample	Decreased	RIA	Atkinson et al., 2008
		Humans	0.22 µm filter	Unfiltered sample	Decreased	EIA	Whembolua et al., 2006
DHEA		Humans	0.2 µm filter	FTC sample	Decreased	EIA	Atkinson et al., 2008
		Humans	0.22 µm filter	Unfiltered sample	Decreased	EIA	Whembolua et al., 2006

Table 4. (continued) Effect of post-collection sample processing on assay results

Target	Analyte	Species	Processing/Treatment	Reference Sample	Effect	Assay	Citation
Other							
Total protein		Humans	0.2 µm filter	FTC sample	No effect	CBQCA[a]	Atkinson et al., 2008
		Humans	Centrifuge - 10,000 x *g* 10 min	FTC sample	No effect	CBQCA[a]	Atkinson et al., 2008
		Humans	0.45 µm	Untreated saliva	Decreased	BCA[b]	Sreebny et al., 1995
		Humans	Centrifuge - 5,000 rpm 20 min	Untreated saliva	Decreased	BCA[b]	Sreebny et al., 1995
		Humans	5 µm filter	Unfiltered sample	Decreased	SDS-PAGE	Ruhl et al., 2011

* Abbreviations: ELISA, enzyme-linked immunosorbent immunoassay; PRRSV, porcine reproductive and respiratory syndrome virus; RT-PCR, reverse transcription polymerase chain reaction; FTC, Freeze-thaw-centrifuge (4,000 x *g* for 10 min); RIA, radioimmunoassay; EIA, enzyme immunoassay; DHEA, dehydroepiandrosterone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

[a] 3-(4-carboxybenzoyl)quinoline-2-carboxalde-hyde (CBQCA) protein quantitation kit (Life Technoloties™, Foster City, CA USA)

[b] BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL USA)

Table 5. Effect of sample storage on the stability of diagnostic analytes

Target	Analyte	Species	Temperature	Storage Time	Effect	Assay	Citation
Total Antibody	IgM	Pigs	≤ 30 °C	12 days	Stable	ELISA	Prickett et al., 2010
	IgA	Humans	-30 °C	12 months	Decreased after 8 months	ELISA	Ng et al., 2003
	" "	Pigs	≤ 20 °C	12 days	Stable	ELSIA	Prickett et al., 2010
	" "	Pigs	30 °C	12 days	Reduced after day 2	ELISA	Prickett et al., 2010
	IgG	Pigs	≤ 20 °C	12 days	Stable	ELISA	Prickett et al., 2010
	" "	Pigs	30 °C	12 days	Reduced after day 2	ELISA	Prickett et al., 2010
Pathogens							
PRRSV	Virus	Pigs	≤ 10 °C	12 days	Stable	RT-PCR	Prickett et al., 2010
	" "	Pigs	20 °C or 30 °C	12 Days	Reduced	RT-PCR	Prickett et al., 2010
	Antibody	Pigs	≤ 10 °C	12 days	Stable	ELISA	Prickett et al., 2010
	" "	Pigs	20 °C or 30 °C	12 Days	Reduced	ELISA	Prickett et al., 2010
Hormones							
17-OHP		Humans	Room Temp	21 days	Reduced after 15 days	RIA	Gröschl et al., 2001
		Humans	4 °C	21 days	Reduced after 15 days	RIA	Gröschl et al., 2001
Cortisol		Humans	Room Temp	90 days	Reduced after one month	RIA	Garde et al., 2005
		Humans	Room Temp	5 days	Stable	RIA	Clements and Parker, 2005
		Humans	Room Temp	7 days	Stable	RIA	Aardal and Holm, 1995
		Humans	Room Temp	10 days	Reduced	RIA	Whembolua et al., 2006
		Humans	5 °C	3 months	Stable	RIA	Garde et al., 2005
		Humans	-20 °C, -80 °C	1 year	Stable	RA	Garde et al., 2005
		Humans	-20 °C	9 months	Stable	RIA	Aardal and Holm, 1995
		Humans	-20 °C	1 freeze-thaw	Reduced	RIA	Aardal and Holm, 1995

Table 5. (continued) Effect of sample storage on the stability of diagnostic analytes

Target	Analyte	Species	Temperature	Storage Time	Effect	Assay	Citation
Hormones							
DHEA		Humans	Room Temp	10 days	Stable	RIA	Whembolua et al., 2006
		Humans	-80 °C	3 freeze-thaws	Stable	RIA	Granger et al., 1999
Testosterone		Humans	4 °C	4 weeks	Weekly linear increase	RIA	Granger et al., 2004
		Humans	Room Temp	10 days	Decreased	RIA	Whembolua et al., 2006
		Humans	Room Temp	14 days	Stable (in males)	RIA	Dabbs, 1991
		Humans	Room Temp	14 days	Increased after 1 week in females	RIA	Dabbs, 1991
		Humans	-20 °C and -40 °C	6 Months	Decreased in high concentration samples	RIA	Granger et al., 2004
		Humans	-20 °C and -40 °C	2 years	Stable in low concentration samples	RIA	Granger et al., 2004
		Humans	-80 °C	2 years	Stable	RIA	Granger et al., 2004
Other							
Genomic DNA		Humans	4 °C	7 days	Reduced	PCR	Ng et al., 2004
		Humans	-70 °C	1 month	Reduced	PCR	Ng et al., 2004

* Abbreviations: ELISA, enzyme-linked immunosorbent immunoassay; PRRSV, porcine reproductive and respiratory syndrome virus; RT-PCR, reverse transcription polymerase chain reaction; 17-OHP, 17-hydroxyprogesterone; RIA, radioimmunoassay; DHEA, dehydroepiandrosterone; PCR, polymerase chain reaction

CHAPTER 2. EFFECT OF COLLECTION MATERIAL ON PIG ORAL FLUID TESTING RESULTS

Submitted for Publication to Research in Veterinary Science

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ABSTRACT

The effect of sampling material, sample processing, and collection order on the detection of analytes in oral fluid was evaluated. Oral fluid samples were collected from 104 pens of commercial wean-to-finish pigs using 3 types of rope. Processed (centrifuged and filtered) and unprocessed oral fluid samples were tested using commercial ELISAs for porcine reproductive and respiratory syndrome virus (PRRSV) antibodies and total IgM, IgA, and IgG. Only unprocessed samples were tested for PRRSV nucleic acid and processed samples for PRRSV neutralizing antibodies. Results were analyzed using a repeated measures ANOVA model and Tukey-Kramer adjusted t-tests. It was found that all three factors could affect results, but the effects were non-uniform and assay-dependent. When testing oral fluid specimens, investigators and diagnosticians should be aware of the potential impact of these factors on specific analytes. For diagnostic submissions, oral fluid samples should be collected using cotton-based materials and undergo minimal post-collection processing.

1. INTRODUCTION

A significant body of research has developed over the last century on the presence of bioanalytical targets in fluids collected from the oral cavity of humans and animals, including antibodies (Coleman and Appleman, 1953; Waldman et al., 1968; Archibald et al., 1987; Parry et

al., 1987; Delaney et al., 2011; Kittawornrat et al., 2012), pathogens (Scott et al., 1997; Wang et al., 2004; Prickett et al., 2008a, 2008b; Kittawornrat et al., 2010; Prickett and Zimmerman, 2010; Detmer et al., 2011; Ramirez et al., 2012), hormones (Vinning and McGinley, 1986; Granger et al., 1999; Malamud and Rodriguez-Chavez, 2011), and drugs (Mucklow et al., 1978; Kidwell et al., 1998; Pink et al., 2009; Meiszberg et al., 2011). Because this area has been extensively researched, nomenclature in the field is well established. Thus, "whole saliva" describes "the fluid obtained … by expectoration" and "oral fluid" is defined as "the fluid obtained by insertion of absorptive collectors in the mouth" (Atkinson et al., 1993).

Consistent with prior research, recent studies in pigs have shown that antibodies (Kittawornrat et al., 2012), pathogens (Pricket et al, 2010; Detmer et al., 2011; Ramirez et al., 2012), and antimicrobials (Meiszberg et al., 2011) can all be detected in oral fluid specimens. In pigs, samples are collected by suspending absorptive material, e.g., rope, in a pen. The animals chew on the material and deposit oral fluid in this process (Prickett et al., 2008a, 2008b; Kittawornrat et al., 2010). The sample is then harvested by compressing the rope. Thus, samples collected from pigs fit the definition of oral fluid specimens.

Although the data is sparse, research has shown that the composition of collection materials may affect the results of human oral fluid testing. For example, the concentration of immunoglobulin A (IgA) (Shirtcliff et al., 2001; Strazdins et al., 2005; Michishige et al., 2006) and M (IgM) (Chang et al., 2009) in oral fluid samples collected with cotton-based materials was lower than IgA and IgM in whole saliva samples from the same persons. Paradoxically, collection of oral fluid using cotton materials reportedly increased (Atkinson et al., 2008) and decreased (Gröschl et al., 2008) testosterone and progesterone levels, respectively. Sample processing has also been shown to affect some assays. Atkinson et al., (2008) reported that filtration of oral fluid samples (0.2µm polyethersulfone membrane filter) resulted in higher concentrations of cortisol, but lower concentrations of testosterone, dehydroepiandrosterone (DHEA), and total protein. Although pig oral fluid samples are increasingly used as a diagnostic specimen, these effects have not been evaluated. Therefore, the objective of this research was to evaluate the effect of oral fluid sampling material and post-collection processing on results produced by antibody- and polymerase chain reaction-based assays.

2. Materials And Methods

2.1 Experimental Design

To compare the effect of sampling material on the detection of analytes in oral fluid, 3 oral fluid samples were successively collected from 104 pens of pigs using 3 different types of ropes, i.e., cotton, hemp, and nylon. To control for the effect of sampling order, pens were sampled in one of 3 sampling sequences: cotton-nylon-hemp; nylon-hemp-cotton; or hemp-cotton-nylon. Following collection, one-half of each oral fluid sample was processed, i.e., centrifuged and filtered, and the other half was left unprocessed. Serum (~5 pigs per pen), and oral fluid samples (processed and unprocessed) were tested for PRRSV antibody and total immunoglobulin for each isotyope (IgM, IgA, IgG). Unprocessed oral fluid samples were tested for PRRSV by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and processed samples for PRRSV neutralizing antibodies. The effect of sampling material, sample processing, and collection order on test results was analyzed using a repeated measures analysis of variance (ANOVA) model and Tukey-Kramer adjusted t-tests. Sample collection protocols and animal use was approved by the Institutional Animal Care and Use Committee (6-11-7164-S).

2.2 Barns and Pigs

Samples were collected from 2 80-pen wean-to-finish barns located in the Midwest USA. For this study, samples were collected from 104 pens; 47 pens from one barn and 57 pens from the other. At the time of collection, each pen contained ~25 pigs weighing 30 to 45 kg (66 – 99lbs). The infection status of the pigs was unknown at the time of sample collection.

2.3 Biological Samples

2.3.2 Oral Fluid

Blood was collected from a convenience sample of ~5 pigs per pen (n = 516) using a single-use blood collection system (Vacutainer®, Becton Dickson, Franklin Lakes, NJ USA) and serum

separation tubes (Kendall, Mansfield, MA USA). Blood samples were allowed to clot, centrifuged for 10 minutes at 1,000 x *g*, aliquotted into 2.0 ml cryogenic vials (Fisher Scientific, Pittsburgh, PA USA), and stored at -70 °C until tested.

2.3.2 Oral Fluid

Oral fluid samples were collected using 45 cm (~18") of 1.27 cm (½") diameter, 3-strand ropes made of cotton (C), hemp (H), or nylon (N). 3 samples were sequentially collected from each pen in one of 3 sampling orders (C-N-H, N-H-C, or H-C-N), with approximately one-third of pens assigned to each order. To collect the sample, a rope was attached to the bars at the front of the pen, thereby allowing the pigs in the pen to interact with the rope and deposit oral fluid. After a 10 minute sampling period, the wet portion of the rope was cut off and placed in a re-sealable plastic bag (Elkay Plastics, Commerce, CA USA). This process was repeated until a sample had been collected from each pen using each rope type.

To recover the oral fluid sample, each bag containing a wet rope was slowly pressed through a hand wringer (BL-44, Dyna-Jet, Overland Park, KS USA) while the oral fluid pooled in the corner of the bag. When a sufficient quantity of fluid had accumulated, the corner of the bag was cut and the oral fluid decanted into a 50 ml centrifuge tube (Fisher Scientific). Samples were subsequently split into 2 aliquots: unprocessed and processed. Unprocessed samples were decanted into 5 ml snap-cap tubes (Becton Dickson) for storage. Processed aliquots were centrifuged at 10,000 x *g* for 2 hours and then filtered using a 0.22µm syringe filter (Fisher Scientific) and placed in 5 ml snap-cap tubes for storage. Both aliquots were stored at -70 °C until assayed.

2.4 Antibody Assays

2.4.1 PRRSV ELISA

Serum and oral fluid (unprocessed and processed) samples were randomly ordered (www.random.org) and then tested for PRRSV antibodies using a commercial ELISA (HerdChek® X3 PRRS ELISA, IDEXX Laboratories, Westbrook, ME USA). Serum was tested

according to the manufacturer's protocol. Oral fluid was tested using a procedure based on the commercial ELISA (Kittawornrat, 2011; Kittawornrat et al., 2012). In brief, the PRRSV oral fluid IgG antibody ELISA was performed by diluting oral fluid samples 1:2 in kit sample diluent and then transferring 250µl of diluted sample onto the antigen plates provided in the kit. Negative and positive kit controls were diluted 1:30 using kit sample diluent and 100µl was added to the plates. Plates were incubated at 4 °C for 16 hours (overnight) and then plates washed 3 times with 400µl of 1X kit wash solution per well. Horse radish peroxidase (HRP)-conjugated antibody was mixed, then added as directed in the modified oral fluid protocol and the plates incubated at 22 °C for 30 minutes. After washing the plates 3 times, 100µl of tetramethylbenzidine (TMB) was added to all wells and incubated for 15 minutes at 22 °C. The color-developing reaction was stopped by pipetting 100µl of kit stop solution to each well and the absorbance was read at 650 nm (EL800, Bio-Tek® Instruments Inc. Winooski, VT USA). Results were reported as sample-to-positive ratios (S/P), with responses ≥ 0.40 considered positive.

2.4.2 PRRSV Neutralizing Antibody

Processed oral fluid samples were tested for PRRSV neutralizing antibody using an indirect immunofluorescence assay previously described for serum samples (Reed and Muench, 1938; Benfield et al., 1992; Christopher-Hennings et al., 2001) and modified for the oral fluid matrix. Unprocessed samples were not suitable for testing. Briefly, oral fluid specimens were treated with ultraviolet (UV) light (254nm) at a distance of 2 inches from the sample for 45 minutes to inactivate viral and bacterial contaminants, then heat treated at 56 °C for 30 min to inactivate bioactive proteins in the sample (Shugars, 1999). Samples were two-fold diluted (1:2 to 1:64) in serum-free Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Thermo Fisher Scientific, Waltham, MA USA) and 100µl of diluted sample transferred to each well of a 96-well, round-bottom cell culture plate (Fisher Scientific). Thereafter, samples were incubated for 2 hours at 37 °C with either 50µl of PRRSV (isolate VR-2332 kindly provided by Dr. Eric Nelson) at a concentration of 1×10^2 median tissue culture infectious dose ($TCID_{50}$) or 50µl of PRRSV (isolate MN-184 kindly provided by Dr. Michael Murtaugh) at a concentration of 2×10^2. After

incubation, 100µl of the resulting suspension was transferred into a 96-well, flat-bottom tissue culture plate (Becton Dickson, Franklin Lakes, NJ USA) containing a confluent monolayer of MARC-145 cells and incubated for 2 hours at 37 °C in a 5% CO_2 incubator. Subsequently, 100µl per well of maintenance medium consisting of DMEM with 2% horse serum (HyClone, Thermo Fisher Scientific) was added, after which the samples were incubated for an additional 48 hours at 37 °C in a 5% CO_2 incubator. Cytopathic effects were visualized by fixing plates with 80% acetone water for 10 minutes at room temperature, then incubating for 2 hours at 37 $^{\circ}C$ with anti-PPRSV nucleocapsid protein specific monoclonal antibody diluted 1:5000 (SDOW17; Rural Technologies, Inc., Brookings, SD USA) and incubating another 2 hours at 37 $^{\circ}C$ with fluorescein fluorophore-conjugated anti-mouse $IgG_{H\&L}$ secondary antibody diluted 1:3000 (Alexa Fluor® 488, Invitrogen, Grand Island, NY USA). Plates were observed under fluorescent microscopy after mounting with 40µl per well of glycerol-PBS (6:4 ratio). The virus neutralization titer (VNT) was determined to be the reciprocal dilution of the sample at which >80% inhibition of the PRRSV cytopathic effect was observed.

2.4.3 Total Immunoglobulin Quantitation

Total immunoglobulin (IgM, IgA, IgG) was quantified in serum and oral fluid (Prickett et al., 2010; Escribano et al., 2011) using isotype-specific commercial ELISA quantitation kits (Bethyl Laboratories, Montgomery, TX USA). Following the manufacturer's instructions, microtitration plates (MaxiSorp^{TM}, Thermo Fisher Scientific) were coated with coating antibody (A100-102A (IgM), A100-100A (IgA), A100-104A (IgG), Bethyl Laboratories) by diluting the antibody in carbonate-bicarbonate buffer (0.05 M /L, pH 9.6) at a rate of 1:100 and adding 100µl to each well. Plates with coating antibody were incubated for one hour at 22 °C and then washed 5 times using 400µl per well (0.05 M Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0, Sigma Aldrich). Plates were blocked using 200µl per well of blocking solution (0.05 M Tris, 0.14 M NaCl, 1.0% BSA, pH 8.0, Sigma Aldrich) and then incubated for 2 hours at 22 °C. Thereafter, the blocking solution was manually emptied from the wells and the plates blotted dry on a paper towel. Blocked plates were dried for 2 hours at 37 °C and stored in a sealed plastic bag at 4 °C until use.

A standard curve was generated on each plate by diluting the provided reference serum (RS10-

107) to the levels suggested by the manufacturer. HRP-conjugated antibody (A100-100P (IgM), A100-102P (IgA), A100-104P (IgG), Bethyl Laboratories) was diluted 1:50,000 for IgM and 1:75,000 for IgA and IgG in sample/conjugate diluent (0.05 M Tris, 0.14 M NaCl, 1.0% BSA, 0.05% Tween 20 pH 8.0, Sigma Aldrich). Optimal serum sample dilutions were determined by performing a 10-fold serial dilution in sample/conjugate diluent. Final serum sample dilutions of 1:10,000 (IgM) and 1:100,000 (IgA, IgG) were used. Optimal oral fluid sample dilutions were determined by testing samples diluted at 1:10, 1:50 and 1:500. Final oral fluid dilutions of 1:50 (IgM, IgG) and 1:500 (IgA) were used. Oral fluid or serum samples that exceeded the range of the standard curve were re-tested at a two-fold higher dilution than originally used.

To complete the assay, 100µl of diluted sample was pipetted onto the appropriate plates and incubated for one hour at 22 °C. Plates were washed 5 times, 100µl of the appropriate HRP-conjugated antibody was added to each well, and incubated for one hour at 22 °C. Plates were washed 5 times and 100µl of TMB was added to each well and incubated at 22 °C. After 15 minutes the reaction was stopped by adding 100µl of stop solution (0.05M sulfuric acid) and the absorbance read at 450 nm (EL800, Bio-Tek® Instruments Inc.).

Two in-house oral fluid control samples were diluted appropriately for each assay and run in duplicate on each plate. The OD results from the control samples were used to calculate the intra-assay (IgM, IgA, IgG) coefficient of variation (CV) using commercial software (Gen5™ data analysis software, Bio-Tek® Instruments Inc.). In this study, an intra-assay CV <10% for each control was deemed acceptable and justified the use of inter-plate averages of known-concentration standards to calculate final immunoglobulin concentrations (Reed et al., 2002).

Antibody concentration in unknown samples was quantified by fitting a 4-parameter logistic regression curve in Gen5™ using the inter-plate average of known-concentration standards. Final concentration (mg/ml) was determined by solving the equation for each unknown and multiplying the resulting value by the dilution rate of the unknown sample.

2.5 Molecular Diagnostics

2.5.1 Nucleic Acid Extraction

Oral fluid samples were extracted using the MagMAX™ pathogen RNA/DNA kit (Applied Biosystems™) and Thermo Electron KingFisher® automated magnetic particle processor. Oral fluid samples were processed using the protocol for "all other sample types". Due to limited sample volume, "processed" samples were not tested

2.5.2 PRRSV RNA Amplification and Detection via Real-Time PCR

Real-time PCR was performed with commercially-available reagent sets (TaqMAN® North American Reagents and TaqMAN® North American Controls, Applied Biosystems™). In this assay, North American PRRSV RNA are reverse-transcribed into cDNA and amplified by Taq® DNA polymerase in a single tube, one-step differential (PRRSV Type 1 vs. Type 2) PCR reaction. Detection of amplified target is accomplished by TaqMAN® hydrolysis probe chemistry. This master mix also contains primers and probes targeting an internal positive control RNA sequence (Xeno™ RNA-01). The internal positive control was spiked into the RT-PCR master mix at a concentration of 100 copies/µl to monitor PCR amplification and allow for detection of failed PCR reactions.

Master mix component volumes per well consisted of 12.5µl of 2X RT-PCR buffer, 2.5µl of 10X PRRSV primer probe mix, 2.5µl of 20X multiplex RT-PCR enzyme mix, and 0.35µl of 100 copies/µl of Xeno™ RNA-01 internal control. Ultimately, 18µl (rounded up) of master mix was combined with 7µl of RNA extract onto a 96-well PCR plate. Real-time RT-PCR was performed (ABI 7500, Applied Biosystems™) using the following cycling conditions: One cycle at 45 °C for 10 min, one cycle at 95 °C for 10 min, 40 cycles of: 97 °C for 2 seconds, 60 °C for 40 seconds. Quality control of the extraction process included negative (nuclease-free water) and positive (PRRSV isolate ISU-P) controls, i.e. nuclease-free water and PRRSV extraction-positive controls. Each 96-well PCR plate included a positive amplification control (TaqMAN® North American PRRSV controls provided by Applied Biosystems™) and a negative amplification control (nuclease-free water). A cycle threshold (Ct) of ≤ 37 was considered

positive for PRRSV.

2.6 Statistical Analysis

Statistical analyses were performed using SAS® Enterprise Guide 4.3 (SAS® Institute, Cary, NC USA). A repeated measures ANOVA model was utilized to analyze the effects of sampling material, post collection processing (centrifugation/filtration), and collection order on antibody assay results. PRRSV antibody ELISA data was log transformed to meet distribution requirements for valid statistical analyses. VNT data was log (base 2) transformed to create a quantitative data set representative of the original two-fold dilution. Total antibody (IgM, IgA, IgG) concentration and PRRSV antibody ELISA evaluations included material, order, processing, and all possible interactions of these factors as fixed effects in the model. VNT data evaluation included material and order as fixed effects. Pen and the "pen by material" interaction were included as random effects in each model. Least square means were calculated and Tukey-Kramer's adjusted t-test was used to determine statistical significance ($p \leq 0.05$) among the factors evaluated.

PRRSV PCR results were analyzed as qualitative (positive/negative) data using a repeated measures logistic regression model. Material and order were included as fixed effects in the model and pen was used as a random effect.

3. RESULTS

Analyses of oral fluid samples for IgM, IgA, and IgG by isotype-specific quantitative assays and analysis of PRRSV-specific diagnostic assays (ELISA, VNT, RT-PCR) are summarized in Table 1 by factor (sample processing, sampling material, collection order). Analyses of 516 serum samples (516 pigs) for IgM, IgA, and IgG showed mean antibody isotype concentrations of 3.55 mg/ml, 1.19 mg/ml, and 11.74 mg/ml, respectively. Comparisons of IgM, IgA, and IgG antibody concentrations in serum and oral fluid specimens are given in Figure 1.

4. DISCUSSION AND CONCLUSIONS

Oral fluid has been described as a diagnostic "mirror of the body" (Mandel, 1993). In contrast to serum, oral fluid collection is "user friendly" both from the perspective of the individual sampled and the one collecting the sample. In pigs, the diagnostic performance of oral fluid-based assays supports the premise that this approach could facilitate improved health monitoring and the surveillance of economically significant pathogens of pigs, e.g., classical swine fever virus, foot-and-mouth disease virus, influenza viruses, PRRSV, porcine circovirus type 2, and others (Corthier and Aynaud, 1977; Eblé et al., 2004; Prickett et al., 2008a, 2008b; Detmer et al., 2011; Kittawornrat et al., 2012; Ramirez et al., 2012). In humans, it has been reported that both the materials used to collect oral fluid specimens and post-collection processing of samples may affect assay results (Shirtcliff et al., 2001; Strazdins et al., 2005; Michishige et al., 2006; Atkinson et al., 2008; Gröschl et al., 2008; Chang et al., 2009). Comparable data involving the collection of oral fluids from animal subjects could not be found in the literature. Therefore, the current study evaluated the effect of three collection materials (cotton, hemp, nylon) and post-collection sample processing (centrifugation and filtration) on oral fluid specimens obtained from 104 pens of ~25 pigs each. Because the experimental design mandated 3 successive sample collections from each pen, pens were sampled in one of 3 sampling sequences (cotton-nylon-hemp; nylon-hemp-cotton; or hemp-cotton-nylon) to control for the effect of sampling order.

Based on the cumulative data (cotton, hemp, nylon) from unprocessed oral fluid samples and serum samples from a subset of pigs (n = 5) from the same pen, the concentration of IgM, IgA, and IgG in oral fluid was 1/237, 1/14, and 1/783, respectively, the concentration in serum. These estimates were similar to previous estimates of total antibody concentrations in oral fluid and serum in human samples (IgM 1/400, IgA 1/10, and IgG 1/800) (Parry et al., 1987). It should be noted that estimates of the variation among pigs could be improved by using serum and oral fluid from individual pigs, rather than pen-based oral fluids and serum samples from a subset of pigs in the pen, as was done in this case. No statistically significant differences in the concentration of IgG (mg/ml) were detected among oral fluid samples collected with cotton, hemp, or nylon rope (Table 1, Figure 1). In contrast, IgM and IgA concentrations differed significantly by collection material, with cotton associated with the lowest antibody concentration estimates.

Non-uniform, but statistically significant differences were also associated with processing, collection order, and the interactions of processing by material. These results are in agreement with prior reports that cotton-based collection materials were associated with lower concentrations of IgA and IgM in oral fluid specimens collected from humans (Shirtcliff et al., 2001; Strazdins et al., 2005; Michishige et al., 2006; Chang et al., 2009). Overall, IgG antibody was the least affected by the factors evaluated. However, the data indicated that sampling material and sample processing should be taken into consideration when collecting oral fluid samples for the specific purpose of detecting IgM or IgA antibodies.

Detection of PRRSV infection in swine populations via testing of oral fluid specimens has become commonplace in North America and elsewhere (Prickett et al., 2008a, 2008b; Kittawornrat et al., 2010; Prickett et al., 2010; Chittick et al., 2011; Kittawornrat et al., 2012; Ramirez et al., 2012). Analysis of the PRRSV antibody ELISA results showed that both the median S/P ratio of the assay and the percentage of positive samples were unaffected by the collection material (Table 1). Consistent with the results from other assays, unprocessed samples and the first of the three samples collected had the highest median ELISA S/P ratios.

The highest rate of PRRS RT-PCR positivity was observed in samples collected with cotton material (Table 1). Prior reports have shown a high correlation between oral fluid and serum PRRSV RT-PCR results based on paired samples from individual animals under experimental conditions (Kittawornrat et al., 2010). These results support the use of cotton material for the collection of pig oral fluids to be tested using PCR-based assays.

Although the PRRSV neutralizing antibody assay has not been validated for the oral fluid matrix, the presence of neutralizing antibodies in human oral fluid samples has been reported for rhinovirus (Douglas et al., 1967), influenza virus (Waldman et al., 1968), mumps (Chiba and Nakao, 1972), cytomegalovirus (Tamura et al., 1980), and herpes virus 7 (Ihira et al., 2003). Likewise, neutralizing antibodies against foot-and-mouth disease virus were detected in buccal fluid samples from cattle and pigs (Figueroa et al., 1973; Archetti et al., 1993; Eblé et al., 2004). In both PRRSV type 2 assays, samples collected with cotton materials produced the lowest antibody titers and samples collected first among the three collections had the highest antibody titers (Table 1). From these data alone, it cannot be determined whether lower antibody titers in

samples collected with cotton were caused by the loss of neutralizing antibody, for example by antibody binding to cotton, or if higher neutralizing antibody titers may have resulted from the presence of virucidal substances in samples collected with other materials.

The objective of this investigation was to evaluate the potential effect of sample collection material, sample processing, and collection order on porcine oral fluid testing. The samples analyzed in this study were collected from commercial wean-to-finish barns without prior knowledge of their health status. It was found that all three factors could affect results, but the effect was non-uniform and assay-dependent. Depending on the populations' level of immune stimulation and infection history, it may be postulated that similar studies in pig populations would produce comparable, if not identical, results. Thus, investigators and diagnosticians should be aware of the potential impact of these factors on specific analytes. In particular, diagnostic oral fluid samples should be collected using cotton-based materials and undergo minimal post-collection processing.

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6. CONFLICT OF INTEREST STATEMENT

Author S. Lizano is employed by IDEXX Laboratories, Inc. The remaining authors declare no conflicting interests with respect to their authorship or the publication of this article.

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Table 1. Effect of collection material, sample processing, and collection order on oral fluid assay results (n = 104 pens)

Factors		Mean Antibody (mg/ml)			Porcine reproductive and respiratory syndrome virus (PRRSV) Assays					
		IgM	IgA	IgG	Antibody ELISA[1]		VN Antibody-1[2]	VN Antibody-2[2]	qRT-PCR (%)	
Material (univariate analysis)										
Cotton	(n = 104)	0.010[b]	0.064[c]	0.012[a]	0.366[a]	(45%)	1:12.4[b]	1:10.1[b]	34[a]	(32.7%)
Hemp	(n = 104)	0.013[a]	0.076[b]	0.013[a]	0.368[a]	(46%)	1:17.9[ab]	1:14.1[b]	5[b]	(4.8%)
Nylon	(n = 104)	0.013[a]	0.081[a]	0.013[a]	0.366[a]	(45%)	1:27.2[a]	1:32.0[a]	2[b]	(1.9%)
Processed[1] (univariate analysis)										
NO	(n = 312)	0.015[a]	0.088[a]	0.015[a]	0.414[a]	(46%)	Not Tested		41	(13.1%)
YES	(n = 312)	0.010[b]	0.059[b]	0.010[b]	0.324[b]	(45%)	1:18.1	1:16.6	Not Tested	
Collection Order (univariate analysis)										
1	(n = 104)	0.014[a]	0.083[a]	0.013[a]	0.388[a]	(45%)	1:21.3[a]	1:19.2[a]	16[a]	(15.4%)
2	(n = 104)	0.012[b]	0.071[b]	0.012[b]	0.350[a]	(46%)	1:17.8[b]	1:16.2[b]	16[a]	(15.4%)
3	(n = 104)	0.011[c]	0.066[b]	0.011[b]	0.334[b]	(45%)	1:15.8[b]	1:14.8[b]	9[a]	(8.7%)
Process x Material										
No x Cotton	(n = 104)	0.012[a]	0.076[b]	0.014[a]	0.420[a]	(45%)	Not tested		34[a]	(32.7%)
No x Hemp	(n = 104)	0.016[a]	0.093[a]	0.015[a]	0.426[a]	(46%)	"	"	5[b]	(4.8%)
No x Nylon	(n = 104)	0.016[a]	0.094[a]	0.015[a]	0.397[a]	(45%)	"	"	2[b]	(1.9%)
Yes x Cotton	(n = 104)	0.008[a]	0.051[e]	0.009[a]	0.318[a]	(45%)	1:12.4[b]	1:10.1[b]	Not Tested	
Yes x Hemp	(n = 104)	0.011[a]	0.059[d]	0.010[a]	0.316[a]	(45%)	1:17.9[ab]	1:14.1[b]	"	"
Yes x Nylon	(n = 104)	0.011[a]	0.068[c]	0.010[a]	0.338[a]	(45%)	1:27.2[a]	1:32.0[a]	"	"

[1] Median PRRSV antibody ELISA S/P values and percent positive samples (IDEXX Laboratories, Inc., Westbrook, ME USA)

[2] Least squares means of virus-neutralizing (VN) antibody titers based on type 2 PRRSV isolates VR-2332 (1) or MN-184 (2).

[3] Centrifuged at 10,000 x *g* for 2 hours and then filtered using a 0.22µm syringe filter (Fisher Scientific, Pittsburgh, PA USA)

[abcde] Groups not sharing a common letter within columns for each factor are statistically different ($p \leq 0.05$)

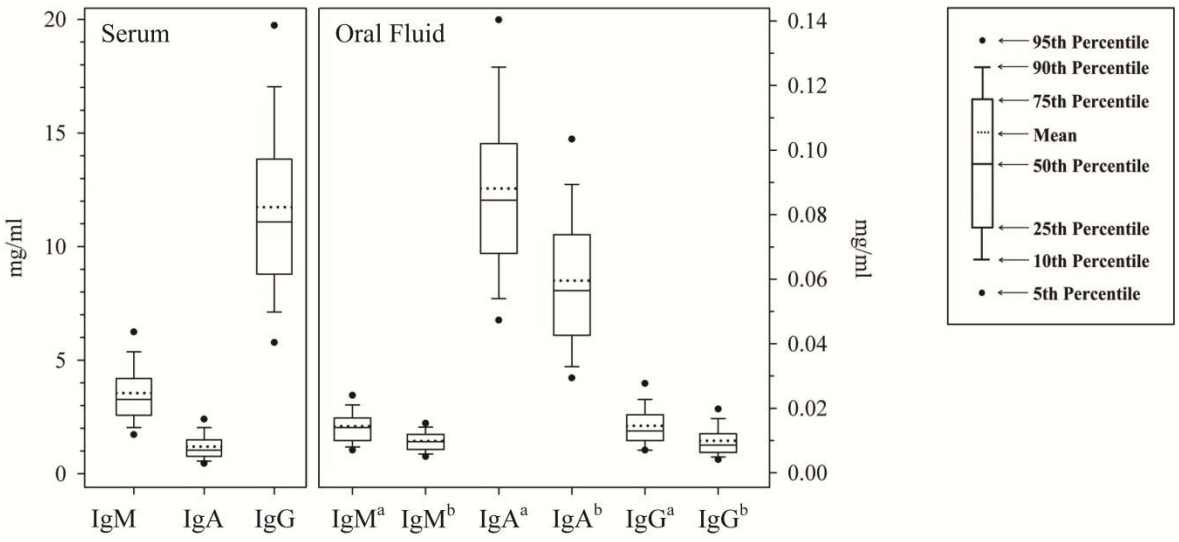

Figure 1. Antibody isotype concentrations in serum, unprocessed[a], and processed[b] oral fluid. Processed oral fluid samples were centrifuged at 10,000 x *g* for 2 hours and then filtered using a 0.22µm filter

CHAPTER 3. PROBABILITY OF DETECTING PRRSV INFECTION IN PEN-BASED SWINE ORAL FLUID

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ABSTRACT

The use of oral fluid specimens for pathogen detection in pig disease surveillance systems has been effective and has helped to overcome some of the challenges faced with collection of individual samples. Pen-based oral fluid sampling allows for the collection of samples that represent a large number of animals, but to be effective, the diagnostic assay must be able to detect low disease prevalence. Five successive oral fluid samples were collected from 25 pens of pigs which consisted of five levels of Porcine reproductive and respiratory syndrome virus (PRRSV) prevalence (0%, 4%, 12%, 20% or 36%). Serum was collected from all pigs in the study to confirm disease prevalence. Serum and oral fluid was tested by six laboratories for PRRSV antibodies using an enzyme-linked immunosorbent assay (ELISA) and PRRSV via reverse transcription-polymerase chain reaction (RT-PCR). Analysis of intra-laboratory agreement for serum and oral fluid assays revealed that RT-PCR is more variable than the PRRSV antibody ELISA. Predicted probability of detection was calculated based on the with-in pen PRRSV prevalence or the level of interaction with the sampling material by PRRSV positive pigs. Probabilities of detection near 100% were achieved with samples collected from high prevalence pens. Overall, this data supports the use of oral fluid in PRRSV surveillance in pig populations and demonstrates its value in comparison to serum samples.

1. INTRODUCTION

Surveillance for pathogens in swine populations became increasingly important when swine production transitioned to confinement-style systems.[8]. The use of oral fluid specimens for pathogen detection in such systems has been effective and has helped to overcome some of the challenges faced with collection of individual samples.[4,6,7,8] Individual sampling can be expensive, labor intensive, and is difficult to obtain a sufficient quantity of samples to represent the entire population. Pen-based oral fluid sampling allows for the collection of a sample representative of a larger number of animals, is convenient, and cost-effective.

A caveat to pen-based oral fluid sampling in disease surveillance is the detection limits of the diagnostic assay. In surveillance programs it is important to have a diagnostic test that can detect low levels of disease prevalence. It has been reported that a porcine reproductive and respiratory syndrome virus (PRRSV) antibody ELISA used for PRRSV surveillance is highly sensitive and specific when testing known status oral fluid samples.[4] Studies testing pig oral fluids for PRRSV by reverse transcription polymerase chain reaction (RT-PCR) are generally in agreement that PRRSV can be readily detected in pen-based samples often times earlier than serum.[3,6,7,8] It has been indicated though that ongoing development is needed to continue to increase the sensitivity of PRRSV RT-PCR oral fluid assays[1,8]. The purpose of this study was to evaluate the detection limits of PRRSV ELISA and RT-PCR assays in pen-based oral fluid samples from commercial swine of known PRRSV prevalence.

2. MATERIALS AND METHODS

2.1 Experimental Design

25 pens of swine in a commercial swine barn were randomly assigned to one of 5 groups. Groups consisted of 0, 1, 3, 5 or 9 vaccinated[a] pigs in a pen (n=25 pigs) of otherwise suspected PRRSV negative pigs. On day 1, the appropriate number of suspected PRRSV negative pigs (n=535) were placed in pens and one oral fluid sample was collected from each pen. On day 2, serum was collected from each of the 535 suspected PRRSV negative pigs to confirm the PRRSV status of all pigs. After serum collection, the appropriate number of

PRRSV vaccinated pigs (*n*=90) were placed into each pen. The following morning (Day 3), 5 successive oral fluid samples (30 minute sampling period, < 10 minutes between samplings) were collected from each pen. During the sampling period, each pen was monitored and the number of vaccinated pigs to contact the sampling rope per minute was recorded ('contact events'). After oral fluid collection, serum samples were collected from each of the vaccinated pigs. Oral fluid (*n*=150) and vaccinated pig serum (*n*=90) were assayed for PRRSV antibodies and PRRS virus each by six diagnostic laboratories. A portion of each expected negative pig serum was pooled into 107 pooled samples (5 pigs per pool). Pooled serum was assayed for PRRS virus by two diagnostic laboratories. All 535 individual serum samples were assayed for PRRSV antibodies by two diagnostic laboratories. Inter-laboratory agreement was measured to compare the assay results from all six laboratories for oral fluid and serum (RT-PCR and ELISA). Predicted probability plots were created using logistic regression based on the probability of detecting a positive (ELISA or RT-PCR) oral fluid sample depending on the within-pen PRRSV prevalence or the number of 'contact events' per sampling period. Sample collection protocols and animal use was approved by the Institutional Animal Care and Use Committee (8-11-7201-S).

2.2 Barns and Pigs

Pigs were housed in commercial swine facilities.[b] On day -11, a group of nursery age pigs were vaccinated intramuscularly with 2 ml of a modified live PRRSV type 2 vaccine.[a] Vaccinated pigs were housed in a separate facility until placed in the trial pens on day 2. Suspected PRRSV negative pigs were housed in similar facilities at a separate location until the trial.

2.3 Biological Samples

Pen-based oral fluid specimens were collected as previously described (Kittawornrat et al., 2012, Prickett et al., 2008). Briefly, a length of 5/8" (~1.6 cm) 3-strand cotton rope (40 inch, ~100 cm) was suspended in the pen such that pigs could actively chew on both loose ends of

the rope. Ropes remained in the pen for 30 minutes, after which the saturated portion of the rope was cut off and sealed in a plastic bag. 5 successive samples were collected from each pen with < 10 minutes between each sampling. 'Contact events' were recorded for each sampling and can be defined as the number of times time a vaccinated pig touched the sampling rope with a maximum of one contact per pig, per minute for a maximum of 30 'contact events' per pig per sampling period. After all samples had been collected, each bag containing saturated ropes was pressed through a hand wringer[c] to extract the oral fluid form the rope. Oral fluid pooled in the corner of the bag, the bag was cut, and oral fluid decanted into 50 ml centrifuge tubes.[d] Samples were aliquotted and stored in 2 ml cryogenic vials[d] at -70 °C until shipped for testing.

Serum was collected using a single-use blood collection system[e] and serum separation tubes.[e] Blood samples were allowed to clot and centrifuged for 10 minutes at 1,000 x g to separate the serum. Pooled samples were created from the 535 suspected PRRSV negative pigs by pooling an equal portion of 5 samples together creating 107 pooled samples. The remaining serum from each of the pigs was stored in individual aliquots. Serum from the 90 PRRSV vaccinated pigs was stored as individual samples. Serum samples were stored in 2 ml cryogenic vials[d], at -70 °C until shipped for testing.

Frozen samples were shipped overnight to participating laboratories in insulated shipping boxes on standard ice packs for diagnostic testing.

2.4 Diagnostic Assays

Serum and oral fluid samples were randomly ordered and tested for PRRSV antibodies and PRRS virus via RT-PCR. Oral fluid (*n*=150) and vaccinated pig serum (*n*=90) samples were each tested by six laboratories for PRRSV antibodies (Labs 1,2,3,6,7,8) and PRRS virus (Labs 1-6). Suspected PRRSV negative serum from individual pigs (n=535) was tested via PRRSV antibody ELISA by two laboratories (Labs 1 and 8). Pools of these samples (5 samples per pool, n=107) were tested by PRRSV RT-PCR by two laboratories (Labs 4 and 5). If a pooled sample tested positive, standard diagnostic protocols were followed and the

individual samples which comprised the respective pool were tested by the same laboratory using the same protocol.

2.4.1 PRRSV antibody ELISA

All laboratories testing serum and oral fluid samples for PRRSV antibodies used the same commercial ELISA assay.[f] Serum was tested according to the manufacturer's protocol. Oral fluid was tested using a procedure based on the commercial ELISA.[2,4] In brief, the PRRSV oral fluid IgG antibody ELISA was performed by diluting oral fluid samples 1:2 in sample diluent provided in the kit, then transferring 250 µl of diluted sample onto the antigen plates provided in the kit. Negative and positive kit controls were diluted 1:30 using sample diluent and 100 µl was added to the plates. Plates were incubated at 4 °C for 16 hours (overnight) and then plates washed 3 times with 400 µl of 1X wash solution per well. Horse radish peroxidase (HRP)-conjugated antibody was mixed, then added as directed in the modified oral fluid protocol and the plates incubated at 22 °C for 30 minutes. After washing the plates 3 times, 100 µl of tetramethylbenzidine (TMB) was added to all wells and incubated for 15 minutes at 22 °C. The color-developing reaction was stopped by pipetting 100 µl of kit stop solution to each well and the absorbance was read[g] at 650 nm. Results were reported as sample-to-positive ratios (S/P), with responses $\geq$ 0.40 considered positive.

2.4.2 PRRSV RT-PCR

Laboratory 1

RNA was extracted from serum[h] and oral fluid[j] using commercial extraction kits and an automated magnetic particle processor.[i] Serum was extracted following the manufacturer instructions, oral fluid was extracted using the protocol for "all other sample types."

Real-time PCR was performed[k] with commercially-available reagent sets[l]. Master mix component volumes per well consisted of 12.5 µl of 2X RT-PCR buffer, 2.5 µl of 10X PRRSV primer probe mix, 2.5 µl of 20X multiplex RT-PCR enzyme mix, and 0.35 µl of 100 copies/ µl of Xeno™ RNA-01 (10,000 copies/µl) internal control. Ultimately, 18 µl

(rounded up) of master mix was combined with 7 µl of RNA extract onto a 96-well PCR plate. The reaction was completed using the following thermal cycling conditions: One cycle at 45 °C for 10 min, one cycle at 95 °C for 10 min, 40 cycles of: 97 °C for 2 seconds, 60 °C for 40 seconds. Quality control of the extraction process included negative (nuclease-free water) and positive (PRRSV isolate ISU-P) controls, i.e. nuclease-free water and PRRSV extraction-positive controls. Each 96-well PCR plate included a positive amplification control[l] and a negative amplification control (nuclease-free water).

Laboratory 2

RNA was extracted from serum samples using a commercial extraction kit[h] and an automated magnetic particle processor[i] following the manufacturer instructions. Oral fluid samples were extracted using a modified version (Protocol "A2", Chittick et al., 2011) of a commercial kit[h]. Lysis/binding solution was prepared by adding 623 µl of carrier RNA to 40 ml of lysis/binding solution, without addition of isopropanol. All other reagents were prepared according to the manufacturer's instructions. 300 µl of oral fluid was added to 450 µl lysis/binding solution in a 1.5 ml microtube. Microtubes were vortexed for 3 minutes then centrifuged at 16,000 x g for 2 minutes. 600 µl of lysate was added to each well of a deep-well plate which contained 350 µl of isopropanol and 20 µl of bead mix.. The lysis plate was loaded onto the automated particle processor[i] along with 2 plates of 300 µl of wash solution 1, 2 plates of 450 µl of wash solution 2, and a final plate of 90 µl of elution buffer. Extraction was then completed using program AM_1836_DW_DV_v3.

Real-time PCR was performed[k] on serum and oral fluid using real-time RT-PCR PRRSV specific reagents for the detection and differentiation of North American & European PRRSV viral RNA (cat #TC-9060-096[m]) following the manufacturer's instructions. The reaction was completed using the following thermal cycling conditions: One cycle at 48 °C for 15 minutes, one cycle at 95 °C for 2 minutes and 45 cycles of 95 °C for 5 seconds and 60 °C for 40 seconds.

Laboratory 3

RNA was extracted from serum[h] and oral fluid[j] samples using an automated particle processor.[n] Serum was extracted following the manufacturer instructions, and oral fluid was extracted following the protocol for "all other sample types".

Real-time PCR was performed[o] with commercially-available reagent sets.[p] Master mix component volumes per well consisted of 12.5 µl of 2X RT-PCR buffer, 2.5 µl of 10X PRRSV primer probe mix, 2.5 µl of 20X multiplex RT-PCR enzyme mix, and 0.35 µl of 100 copies/ µl of Xeno™ RNA-01 (10,000 copies/µl) internal control. Ultimately, 18 µl of master mix (rounded up) was combined with 7 µl of RNA extract onto a 96-well PCR plate. The reaction was completed using the following thermal cycling conditions: One cycle at 45 °C for 10 min, one cycle at 95 °C for 10 min, 40 cycles of: 95 °C for 2 seconds, 60 °C for 45 seconds.

Laboratory 4

RNA was extracted from serum and oral fluid samples using modified versions of a commercial kit[h] and a automated particle processor.[n]

For serum, lysis/binding solution was prepared by combining the lysis/binding solution concentrate from the kit (part# 8500G of AM1836[q]) with a lysis/binding solution concentrate not provided in the kit (cat# AM8500[q]), isopropanol was not added at this time. 2 µl of carrier RNA was then added to 350 µl of the prepared lysis/binding solution concentrate, and 350 µl 100% isopropanol per reaction. Wash solution 1 was prepared by adding 12 ml 100% isopropanol to the wash solution 1 concentrate provided in the kit (part# 8504G of AM1836[q]), and adding 70 ml 100% isopropanol to an additional wash solution 1 concentrate, not provided in the kit (cat# AM8504[q]). Both prepared wash solution 1's were then combined to create the final wash solution 1. Wash solution 2 was prepared by adding 32 ml of 100% ethanol to the wash solution 2 concentrate provided in the kit (part# 8640G of AM1836[q]) and adding 160 ml of 100% ethanol to a wash solution 2 concentrate not provided in the kit (cat# AM8640[q]). Both prepared wash solution 2's were then combined to create

the final wash solution 2. 300 µl of serum was added to 700 µl of prepared lysis/binding solution in a deep-well plate. The lysis plate was loaded onto the automated particle processor[n] along with 2 plates of 300 µl of wash solution 1, 2 plates of 450 µl of wash solution 2, one plate of 90 µl of elution buffer and a deep well tip comb in a deep well plate. Extraction was then completed using program AM1836_DW_HV_v3.

For oral fluid, lysis/binding solution was prepared by adding 2 µl of carrier RNA and 2 µl of Xeno[TM] RNA[q] (1,000 copies/ µl) to 450 µl of lysis/binding solution per reaction, without addition of isopropanol. All other reagents were prepared according to the manufacturer's instructions. 300 µl of oral fluid was added to 450 µl lysis/binding solution in a deep-well plate. Plates were sealed and vortexed at max speed on a plate shaker for 5 min, then centrifuged at 3,300 x g for 5 min. 600 µl of lysate was added to each well of a new deep-well plate which contained 350 µl of isopropanol and 20 µl of bead mix. The lysis plate was loaded onto the automated particle processor[n] along with 2 plates of 300 µl of wash solution 1, 2 plates of 450 µl of wash solution 2, one plate of 90 µl of elution buffer and a deep well tip comb in a deep well plate. Extraction was then completed using program AM1836_DW_300v2.

Real-time PCR was performed[k] with commercially-available reagent sets.[l] Master mix component volumes per well consisted of 12.5 µl of 2X RT-PCR buffer, 2.5 µl of 10X PRRSV primer probe mix, 2.5 µl of 20X multiplex RT-PCR enzyme mix, and 0.5 µl nuclease-free water. Ultimately, 18 µl of master mix was combined with 7 µl of RNA extract onto a 96-well PCR plate. The reaction was completed using the following thermal cycling conditions: One cycle at 48 °C for 10 min, one cycle at 95 °C for 10 min, 40 cycles of: 95 °C for 2 seconds, 60 °C for 70 seconds.

Laboratory 5

RNA was extracted from serum and oral fluid samples using a commercial kit[j] and automated particle processor[r]. Serum was extracted following the manufacturer instructions for low-cell-content samples. Oral fluid samples were extracted using the protocol for "all

other sample types". 300 µl of sample (serum or oral fluid) was used in the reaction and reagents were prepared accordingly with one exception: in place of 2 µl of Xeno™ RNA[q] (1,000 copies/ µl) control in the lysis/binding solution, 8 µl of in-house control was used. Extraction of both sample types was completed using program 4462359_DW_HV..

Real time RT-PCR was performed[k] using an in-house PRRSV RT-PCR assay (cat #TC-9060-096[m]). The assay covers two target regions of the PRRSV Type 1 and 2 genes using FAM as a reporter dye for the detection of the Type 1 PRRSV and TAMRA as a reporter dye for the detection of the Type 2 PRRSV. CY5 was used as a reporter dye for the detection of the extraction/inhibition control.

Serum component volumes per well included 19.25 µl of master mix (includes buffer, primer and probes), 0.25 µl of enzyme 1, 0.5 µl of enzyme 2, and 8 µl of extracted serum sample or internal control. Oral fluid component volumes per well included 16.25 µl of master mix (includes buffer, primer and probes), 0.25 µl of enzyme 1, 0.5 µl of enzyme 2, and 8 µl of extracted oral fluid sample or internal control. Plates were briefly vortexed (10 sec) and centrifuged before being loaded onto the thermocycling instrument. The reaction was completed using the following thermal cycling conditions: One cycle at 48 °C for 15 min, one cycle at 95 °C for 2 min, 45 cycles of: 95 °C for 5 seconds, 60 °C for 40 seconds

Laboratory 6

RNA was extracted from serum and oral fluid using a commercial kit[h] and automated particle processor.[n] Serum was extracted following manufacturer's instructions, oral fluid was extracted using a modified protocol. Briefly, lysis/binding solution was prepared by mixing 235 µl of lysis/binging solution concentrate (cat# AM8500 - not provided in the kit) with 3 µl of carrier RNA (1 µg/µl) per sample to be extracted. Remaining reagents were prepared as directed by the manufacturer. 20 µl of bead mix was added to each well of a deep-well plate containing lysis/binding solution. 300 µl of oral fluid was then added to each well of the same plate followed by 170 µl of 100% isopropanol. The rest of the extraction procedure was completed as directed by the manufacturer. Extraction was completed using the program

AM1836_DW_saliva.

Real-time PCR was performed[k] with commercially-available reagent sets.[l] Master mix component volumes per well consisted of 12.5 µl of 2X RT-PCR buffer, 2.5 µl of 10X PRRSV primer probe mix, 2.5 µl of 20X multiplex RT-PCR enzyme mix, and 0.5 µl Xeno™ RNA[q] (1,000 copies/ µl) control. Ultimately, 18 µl of master mix was combined with 7 µl of RNA extract onto a 96-well PCR plate. Loaded plates were centrifuged at 2,000 rpm for 5 seconds. The reaction was completed using the following thermal cycling conditions: One cycle at 48 °C for 10 min, one cycle at 95 °C for 10 min, 40 cycles of: 97 °C for 2 seconds, 60 °C for 40 seconds.

2.5 Statistical Analysis

Intra-laboratory agreement of oral fluid and serum assay results (ELISA and RT-PCR) between all laboratories was compared using Cochran's Q and McNemar's test.[s] The predicted probability of a positive oral fluid test (ELISA or RT-PCR) based on the within-pen PRRSV prevalence or the number of 'contact events' within each pen was calculated using logistic regression[t]. In the logistic model, binary assay results (ELISA or RT-PCR) were modeled as the response variable and 'contact events' or within-pen prevalence as the explanatory variable. Contact events were grouped by every ten events from 1-100. The resulting probabilities and 95% confidence intervals were plotted in Sigma Plot 11.0[u]

3. RESULTS

Testing of the 535 expected PRRSV negative pigs revealed one ELISA positive pig. Upon re-test the sample remained ELISA positive but was RT-PCR negative. This pen was omitted from the ELISA data analysis because it could not be accounted for in the contact events for this pen. 2 of the 107 pooled expected negative serum samples tested RT-PCR positive by one laboratory. Individual samples from the respective pools were tested by the same laboratory using the same protocols and were all negative. Therefore the samples were considered PRRSV negative for all analyses.

3 vaccinated pig serum samples tested PRRSV antibody ELISA negative by all six laboratories and 1 vaccinated pig serum sample tested PRRSV RT-PCR negative by all six laboratories. Within-pen PRRSV prevalence and total 'contact events' were adjusted to reflect the number of vaccinated pigs that tested positive by PRRSV antibody ELISA or PRRSV RT-PCR by at least one of six laboratories.

Intra-laboratory agreement of PRRSV RT-PCR oral fluid testing resulted in greater laboratory variation than PRRSV antibody ELISA oral fluid testing (Tables 2, 3, 4 and 5). Analysis of intra-laboratory agreement between individual laboratories showed significant differences in ten comparisons for oral fluid RT-PCR (Table 4) and six for PRRSV antibody oral fluid ELISA.

Predicted probability plots (Figure 1 and 2) showed comparable probabilities of detection between the two assays. The probability of detection achieved near 100% with 36% within-pen PRRSV prevalence or ≥ 90 'contact events' per sampling period.

4. DISCUSSION

As evidenced by the variation in RT-PCR results among laboratories, improvements need to be made to further standardize such assays. Data were analyzed categorically (positive or negative) because no methods exist to standardize continuous quantitative results. The PRRSV antibody ELISA contains internal kit controls that are used to calculate a sample to positive ratio, which helps reduce plate-to-plate and lab-to-lab variation. Similar methods may benefit future RT-PCR assays.

Despite inconsistencies in quantitative data, both assays showed promising categorical results. Predicted probability plots showed predictions near 100% for higher prevalence levels and numbers of 'contact events'. Predicted probabilities were also significantly better than the scenario in which one randomly selected pig were bled and tested on either assay. Even if the assumption is that the assays are 100% sensitive and specific, the probability of detection is only ~35%.

For surveillance purposes, detection of low prevalence populations is important. To achieve

better detection the behavior of pigs in the pen must be considered. It has been reported that in a 20 minute sampling period ~70% of pigs in a pen of ~20 pigs will interact with the sampling material if one rope is presented to the pen. If 2 ropes are presented to the pen, ~90% of pigs were reported to interact with the sampling material.[9] In this study, vaccinated pigs were introduced into study less than 24 hours prior to oral fluid collection. It is likely, that the estimates in this report are conservative because the vaccinated pigs had not established social order with in the pen, and may not have interacted with the sampling material as much as in a typical setting. As seen in Figures 1 and 2, predicted probability increases dramatically with increased 'contact events'. Even in low-prevalence situations, if more rope interaction is elicited, better detection may occur.

This research supports the continuation of studies of oral fluid diagnostics and surveillance in veterinary medicine. Standardized diagnostic methods, a better understanding of pig social behavior and refined sample collection protocols will all help to support the ever-changing landscape of pig production.

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6. SOURCES AND MANUFACTURERS

[a] Ingelvac PRRS® MLV, Boehringer Ingelheim Vetmedica, Inc. St. Joseph, MO

[b] Murphy-Brown LLC, Western Operations, Ames, IA

[c] BL-44, Dyna-Jet, Overland Park, KS

[d] Fisher Scientific, Pittsburgh, PA

[e] Vacutainer®, Becton Dickson, Franklin Lakes, NJ

[f] HerdChek® X3 PRRS ELISA, IDEXX Laboratories, Westbrook, ME

[g] EL800, Bio-Tek® Instruments Inc. Winooski, VT

[h] Ambion® MagMAX™ -96 Viral RNA Isolation kit, Applied Biosystems™, Foster City, CA

[i] KingFisher® 96 Thermo Fisher Scientific, Waltham, MA

[j] Ambion® MagMAX™ pathogen RNA/DNA kit Applied Biosystems™, Foster City, CA

[k] ABI 7500 Fast, Applied Biosystems™, Foster City, CA

[l] VetMAX™ TaqMAN® NA and EU and TaqMAN® NA and EU Controls, Applied Biosystems™, Foster City, CA

[m] Tetracore®, Rockville, MD

[n] MagMAX™ Express-96 Magnetic Particle Processor, Applied Biosystems™, Foster City, CA

[o] Roche Lightcycler 480, Roche Applied Science, Indianapolis, IN

[p] Ag-Path PRRS PCR kit, Applied Biosystems™, Foster City, CA

[q] Applied Biosystems™, Foster City, CA

[r] BioSprint 96 Workstation, Qiagen, Valencia, CA

[s] MedCalc 9.2.1.0, MedCalc Software, Mariakerke, Belgium

[t] SAS version 9.2, SAS Institute Inc., Cary, NC

[u] Systat Software, Inc. San Jose, CA

7. DECLARATION OF CONFLICTING INTERESTS

Author S. Lizano is employed by IDEXX Laboratories Inc., author Rolf Rauh is employed by Life Technologies (Applied Biosystems™), and author Rohan Shah is employed by Tetracore. The remaining authors declare no conflicting interests with respect to their authorship or the publication of this article.

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Table 1. Vaccinated pig serum (n = 90) RT-PCR and ELISA results, Chochran's Q p-value of ≤ 0.05 indicates significant difference between the testing results from six laboratories

	Percent and Number of Samples to Test PRRSV Positive								Cochran's Q p-value
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	
RT-PCR	93% (84/90)	93% (80/86)*	93% (84/90)	91% (82/90)	99% (89/90)	97% (87/90)	DNT	DNT	0.012
ELISA	97% (87/90)	97% (87/90)	97% (87/90)	DNT	DNT	96% (86/90)	96% (83/86)*	96% (83/86)*	0.416

*Missing samples

Table 2. Oral fluid (n = 150) RT-PCR results, Chochran's Q p-value of ≤ 0.05 indicates significant difference between the testing results from six laboratories

PRRSV Prevalence	n	Percent and Number of Samples to Test RT-PCR Positive												Cochran's Q p-value
		Lab 1		Lab 2		Lab 3		Lab 4		Lab 5		Lab 6		
0%	50	0%	(0/50)	0%	(0/50)	0%	(0/50)	2%	(1/50)	2%	(1/50)	2%	(1/50)	0.700
4%	25	12%	(3/25)	16%	(4/25)	12%	(3/25)	8%	(2/25)	20%	(5/25)	20%	(5/25)	0.443
8%	5	80%	(4/5)	100%	(5/5)	80%	(4/5)	20%	(1/5)	100%	(4/5)	80%	(4/5)	0.015
12%	20	85%	(17/20)	95%	(19/20)	55%	(11/20)	55%	(11/20)	100%	(20/20)	90%	(18/20)	<0.001
20%	25	72%	(18/25)	88%	(22/25)	40%	(10/25)	32%	(8/25)	80%	(20/25)	72%	(18/25)	<0.001
36%	25	96%	(24/25)	96%	(24/25)	76%	(19/25)	76%	(19/25)	100%	(25/25)[a]	96%	(24/25)	0.002

Table 3. Oral fluid (n = 145) ELISA results, Chochran's Q p-value of ≤ 0.05 indicates significant difference between the testing results from six laboratories

PRRSV Antibody Prevalence	n	Percent and Number of Samples to Test ELISA Positive												Cochran's Q p-value
		Lab 1		Lab 2		Lab 3		Lab 6		Lab 7		Lab 8		
0%	50	2%	(1/50)	0%	(0/50)	0%	(0/50)	0%	(0/50)	0%	(0/50)	0%	(0/50)	0.416
4%	25	24%	(6/25)	16%	(4/25)	12%	(3/25)	8%	(2/25)	24%	(6/25)	8%	(2/25)	0.067
8%	2	0%	(0/5)	0%	(0/5)	0%	(0/5)	0%	(0/5)	0%	(0/5)	0%	(0/5)	1.000
12%	15	67%	(10/15)	60%	(9/15)	80%	(12/15)	47%	(7/15)	73%	(11/15)	53%	(8/15)	0.048
20%	20	68%	(17/25)	88%	(22/25)	92%	(23/25)	80%	(20/25)	92%	(23/25)	88%	(22/25)	0.015
32%	10	90%	(9/10)	100%	(10/10)	90%	(9/10)	90%	(9/10)	100%	(10/10)	90%	(9/10)	0.722
36%*	15	87%	(13/15)	93%	(14/15)	93%	(14/15)	87%	(13/15)	100%	(15/15)	87%	(13/15)	0.352

Table 4. Intra-laboratory agreement of RT-PCR results, McNemar's test *p*-value of ≤ 0.05 indicates significant difference between laboratories

Laboratory	1	2	3	4	5	6
1	·	0.039	0.0001	<0.0001	0.013	0.344
2	·	·	<0.0001	<0.0001	0.754	0.424
3	·	·	·	0.405	<0.0001	<0.0001
4	·	·	·	·	<0.0001	<0.0001
5	·	·	·	·	·	0.180
6	·	·	·	·	·	·

Table 5. Intra-laboratory agreement of oral fluid ELISA results, McNemar's test *p*-value of ≤ 0.05 indicates significant difference between laboratories

Laboratory	1	2	3	6	7	8
1	·	0.648	0.302	0.359	0.078	0.804
2	·	·	0.727	0.008	0.180	0.180
3	·	·	·	0.002	0.388	0.016
6	·	·	·	·	0.0001	0.375
7	·	·	·	·	·	0.003
8	·	·	·	·	·	·

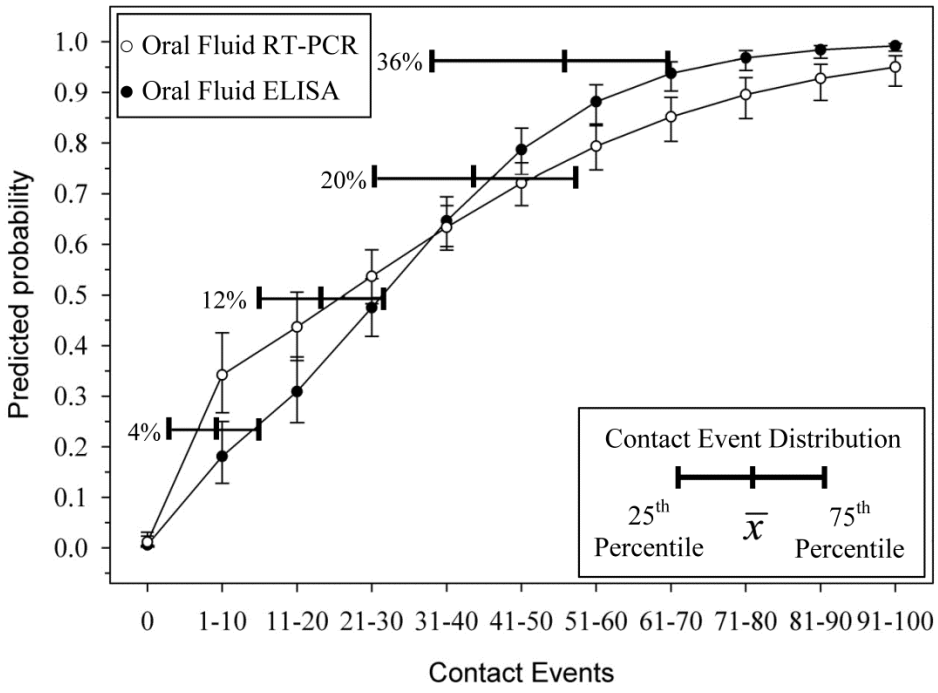

Figure 1. Predicted probability (mean and 95% confidence interval) of detecting a PRRSV positive (ELISA or RT-PCR) oral fluid sample by number of 'contact events' and relative distribution of 'contact events' by expected PRRSV prevalence

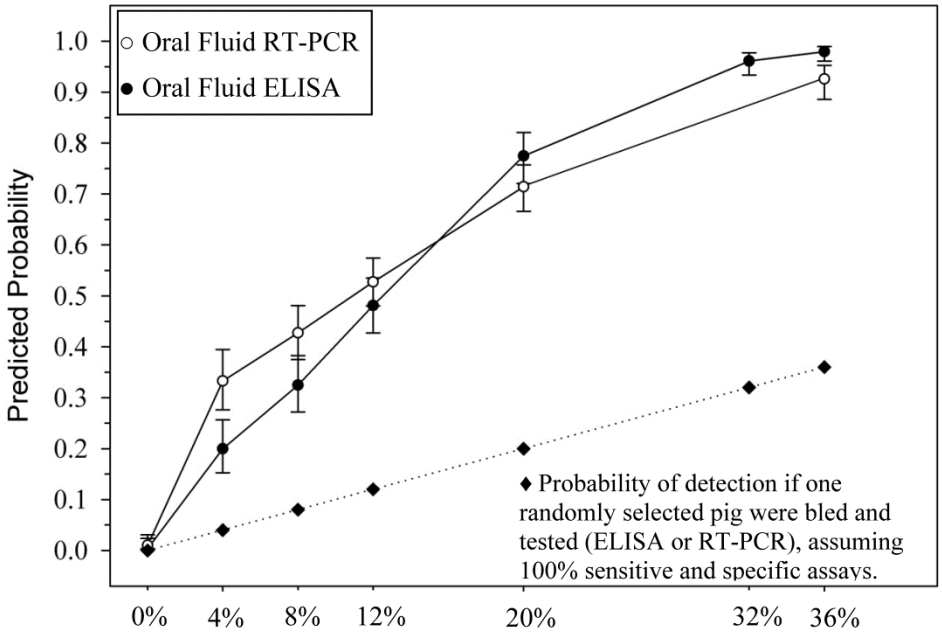

Figure 2. Predicted probability (mean and 95% confidence interval) of detecting a PRRSV positive (ELISA or RT-PCR) oral fluid sample by within-pen PRRSV prevalence, and the probability of detection if one randomly selected pig in a pen were bled and tested (ELISA or RT-PCR), assuming 100% sensitive and specific assays.

GENERAL CONCLUSIONS

The transition of animal production to large-scale confinement- or herd-based systems has created opportunities for disease transmission that at one time did not exist. Increased livestock transportation, frequent human-animal contact, the frequency of human travel, and dense human populations create countless opportunities for disease outbreak and spread. Because of these challenges, it is important to utilize scientifically-based diagnostic and surveillance programs to monitor the disease state of animal populations. Oral fluid samples have shown to be a useful biological specimen in surveillance of swine pathogens. Pen-based oral fluid specimens can represent a large number of animals with fewer samples than serum or other sample types. Moreover, oral fluid collection is non-invasive, user-friendly, and can be done on a regular basis.

As research on oral fluid use developed, it became apparent that many factors can affect the results of specimen testing. The research presented in this thesis reviewed the current knowledge of the external factors that can adversely affect oral fluid diagnostics. In summary, collection material, sample processing and sample storage can have an impact on testing for antibodies, pathogens, hormones and proteins. In the first research chapter presented in this thesis, the effect of sample collection material and post-collection sample processing was investigated in pig oral fluid samples. In searching the literature no other reports have been produced on this topic. Though, it was not within the scope of this project to elucidate the mechanism(s) responsible for the observed effects, it is clear that there is a need for additional research to investigate the present findings.

Because pig oral fluid samples are commonly collected from pens of animals, it is important to understand the limitations of the diagnostic assays being utilized. The purpose of a surveillance program is to detect infection in the early stages. To accomplish this successfully, the diagnostic assay must be able to detect low disease prevalence within the population being sampled. The second research project presented in this thesis examined the ability of commercial assays to detect low levels of PRRSV prevalence in a commercial production setting. From the results of this study, it is apparent that disease prevalence is only one factor on detecting infection in pen-based samples. The social behavior of pigs

largely influences how frequently each pig in the pig interacts with the sampling material. Though the disease prevalence may seemingly be well within the limits of the diagnostic assay, if the infected pigs don't contribute to the sample, infection may not be detected. There is a need for further research to understand this aspect of pen-based sampling and further improve oral fluid diagnostics.

All things considered, the value of oral fluid specimens in veterinary and human medicine, rationalizes investments in future research to continue discovering how this unique specimen can be used to its full potential.